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## Production of a Class II Two-Component Lantibiotic of *Streptococcus pneumoniae* Using the Class I Nisin Synthetic Machinery and Leader Sequence<sup>▽</sup>

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**Recent studies showed that the nisin modification machinery can successfully dehydrate serines and threonines and introduce lanthionine rings in small peptides that are fused to the nisin leader sequence. This opens up exciting possibilities to produce and engineer larger antimicrobial peptides *in vivo*. Here we demonstrate the exploitation of the class I nisin production machinery to generate, modify, and secrete biologically active, previously not-yet-isolated and -characterized class II two-component lantibiotics that have no sequence homology to nisin. The nisin synthesis machinery, composed of the modification enzymes NisB and NisC and the transporter NisT, was used to modify and secrete a putative two-component lantibiotic of *Streptococcus pneumoniae*. This was achieved by genetically fusing the propeptide-encoding sequences of the *spr1765* (*pneA1*) and *spr1766* (*pneA2*) genes to the nisin leader-encoding sequence. The chimeric prepeptides were secreted out of *Lactococcus lactis*, purified by cation exchange fast protein liquid chromatography, and further characterized. Mass spectrometry analyses demonstrated the presence and partial localization of multiple dehydrated serines and/or threonines and (methyl)lanthionines in both peptides. Moreover, after cleavage of the leader peptide from the prepeptides, both modified propeptides displayed antimicrobial activity against *Micrococcus flavus*. These results demonstrate that the nisin synthetase machinery can be successfully used to modify and produce otherwise difficult to obtain antimicrobially active lantibiotics.**

Small antimicrobial peptides produced by Gram-positive bacteria are named bacteriocins. One group of bacteriocins, the nonlantibiotics, comprises peptides that do not require modification for their antimicrobial activity (46). Members of another group, the lantibiotics, require posttranslational modifications to acquire biological activity (11, 47). Lantibiotics are produced as inactive prepeptides, consisting of an N-terminal leader peptide and a C-terminal propeptide part. Most of the serine and threonine residues of the propeptide are dehydrated to dehydroalanine (Dha) and dehydrobutyrine (Dhb), respectively, by LanB- or LanM-type enzymes (“Lan” is a general abbreviation for proteins involved in lantibiotic biosynthesis). LanC or LanM enzymes can subsequently couple these dehydroresidues to cysteines, thus forming a (methyl)lanthionine ring. After the leader peptide is removed from the prepeptide by the extracellular LanP or transmembrane LanT proteins, the active lantibiotic is released. The immunity against the produced lantibiotics is provided by the LanI and/or LanFEG proteins (3, 21, 41). Three classes of lantibiotics are distinguished (47). Class I lantibiotics are modified by

two enzymes, LanB and LanC. In class II lantibiotics, dehydration and cyclization are performed by a single enzyme called LanM. The C-terminal sequences of LanM-type enzymes share homology with the LanC proteins. LanM enzymes share no homology with the LanB proteins (36, 45). Class II also includes two-component lantibiotics, of which antimicrobial activity depends mainly on the synergistic action of both peptides (33, 34). Each of the peptides of the two-component lantibiotics, except cytolysin, possesses its own dedicated modification LanM enzyme. Class III consists of lantibiotics with functions other than antimicrobial activity (17, 48).

Due to an increasing resistance of bacteria to available antibiotics, there is an urgent need to search for substances active against multidrug resistant pathogens. Since some lantibiotics exhibit a stable activity at nanomolar concentrations against antibiotic-resistant pathogens, it is currently of great interest to apply lantibiotics (6, 32, 40). It has already been shown with a mouse model that mersacidin is active against methicillin-resistant *Staphylococcus aureus* (MRSA) strains (18). Another lantibiotic, lactacin 3147, is a successful antimicrobial agent against MRSA, vancomycin-resistant *Enterococcus faecalis*, penicillin-resistant *Streptococcus pneumoniae*, *Propionibacterium acnes*, and *Streptococcus mutans* (8).

One of the most studied lantibiotics is nisin (9, 10, 22, 27), a class I lantibiotic produced by certain *Lactococcus lactis* strains. It has a long record of safe industrial use as a food preservative (6). Due to the broad activity spectrum against Gram-positive pathogens, including *S. pneumoniae*, nisin has good potential for a number of other applications (9). Re-

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TABLE 1. Strains and plasmids used in this study

Strain/plasmid	Description <sup>b</sup>	Reference or source
<b>Strains</b>		
<i>S. pneumoniae</i> R6	D39 ( $\Delta cps2$ 2538–9862) with increased transformation efficiency	14
<i>L. lactis</i> NZ9000	MG1363 $\Delta pepN::nisRK$	15
<i>S. aureus</i> RN6390B		Lab collection
<i>S. mitis</i> NTCC10712		Lab collection
<i>E. faecalis</i> V583		42
<i>M. flavus</i> NIZO B423		NIZO food research <sup>a</sup>
<b>Plasmids</b>		
pIL3BTC	<i>nisBTC</i> , encoding nisin modification machinery; Ery <sup>r</sup>	39
pNZ8048	Nisin-inducible <i>PnisA</i> ; Cm <sup>r</sup>	7
pNG8048E	Nisin-inducible <i>PnisA</i> , pNZ8048 derivative containing Ery <sup>r</sup> gene to facilitate cloning; Cm <sup>r</sup> Ery <sup>r</sup>	Lab collection
pNZnisA-E3	<i>nisA</i> , encoding nisin	19
pNZE3-nis-spr1765	Contains <i>nisA</i> gene and <i>SPR1765</i> gene	This work
pNZE3-nis-spr1766	Contains <i>nisA</i> gene and <i>SPR1766</i> gene	This work
pNZE3-spr1765	Contains a part of <i>nisA</i> gene which encodes leader peptide of nisin and leaderless part of <i>SPR1765</i> gene fused in frame	This work
pNZE3-spr1766	Contains a part of <i>nisA</i> gene which encodes leader peptide of nisin and leaderless part of <i>SPR1766</i> gene fused in frame	This work
pNGspr1765-1766	pNG8048E contains <i>spr1765</i> and <i>spr1766</i> genes under own promoter	This work

<sup>a</sup> Dutch Institute of Dairy Research.<sup>b</sup> Ery<sup>r</sup>, erythromycin resistance; Cm<sup>r</sup>, chloramphenicol resistance.

cently, it was shown that designed hexapeptides and nonlantibiotic peptides fused to the leader peptide of nisin could be successfully modified by NisB and NisC and exported out of *L. lactis* via NisT (16, 20, 38). The discovery that the lantibiotic modification enzymes LanB, LanC, and LanM possess rather low substrate specificities brings a new opportunity to use them as a tool to improve the stability and activity of peptides potentially valuable for medical applications (2, 16, 39).

Here, we present the successful application of the nisin expression/modification system to produce, modify, and secrete entirely unrelated putative lantibiotics that, based on bioinformatic predictions, belong to the class II lantibiotics. The produced peptides were dehydrated multiple times, as shown by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry. Importantly, the modified peptides showed antimicrobial activity against *Micrococcus flavus*. Our study demonstrates that the nisin production/modification machinery can be used to produce and posttranslationally modify silent lantibiotics, i.e., those for which production conditions are not known, that otherwise would be difficult to obtain from their natural sources.

#### MATERIALS AND METHODS

**Bacterial strains and growth conditions.** Strains and plasmids used in this study are listed in Table 1. Strains were stored in 10% (vol/vol) glycerol at –80°C. *Streptococcus pneumoniae*, *Enterococcus faecalis*, *Staphylococcus aureus*, and *Streptococcus mitis* strains were grown at 37°C in standing M17 (Difco) broth supplemented with 0.5% (wt/vol) glucose (GM17) and, when appropriate, 2 µg/ml chloramphenicol. *Lactococcus lactis* and *Micrococcus flavus* were grown at 30°C in GM17 or minimal medium (39) supplemented with 5 µg/ml chloramphenicol and/or 5 µg/ml erythromycin when appropriate.

**Construction of chimeric peptides.** Standard genetic manipulations were performed essentially as described by Sambrook et al. (43). Plasmid pIL3BTC encoding the nisin modification machinery (39) and plasmid pNZnisA-E3 (19) were used to produce and modify chimeric peptides. Briefly, two open reading frames, *spr1765* and *spr1766* (*pneA1* and *pneA2*, respectively), were amplified by PCR from genomic DNA of *S. pneumoniae* R6 and cloned into pNG8048E, resulting in a plasmid, named pNGspr1765-1766, for which *L. lactis* NZ9000 (39)

was used as a host. All the subsequent genetic cloning procedures were performed with this organism. This plasmid, pNGspr1765-1766, served as a template to amplify separately the genes *spr1765* and *spr1766*. Subsequently, each of the amplified products of genes *spr1765* and *spr1766* was subcloned to the pNZnisA-E3 expression plasmid. This resulted into two new plasmids, pNZE3-nis-spr1765 and pNZE3-nis-spr1766, which carried the nisin structural gene and a lantibiotic gene next to one another. To construct genetic fusions of the nisin leader sequence and the structural leaderless sequence of the *spr1765* and *spr1766* (*pneA1* and *pneA2*, respectively) genes in frame, the round PCR method with 5'-phosphorylated primers was used as described previously (39), using Phusion DNA polymerase (Finnzymes). The final chimeric peptide expression plasmids pNZE3-spr1765 and pNZE3-spr1766 were thus constructed. These plasmids were used separately in combination with plasmid pIL3BTC to produce and secrete modified chimeric peptides. Plasmid isolation was performed by means of the plasmid DNA isolation kit (Roche Applied Science). Restriction analysis was performed with restriction enzymes from Fermentas. DNA ligation was performed with T4 DNA ligase (Fermentas).

**Peptides.** Peptides encoding the sequence of leaderless P<sub>n</sub>eA1 and P<sub>n</sub>eA2 were purchased from Pepscan Lelystad NL. Peptides were purified to homogeneity by high-pressure liquid chromatography (HPLC) on a Jupiter Proteo C<sub>12</sub> (4-µm, 90-Å, 250- by 4.6-mm) column with an acetonitrile gradient.

Expression and purification of microbially produced peptides were performed as follows. Overnight cultures of *L. lactis* NZ9000 containing pIL3BTC and a chimeric peptide expression plasmid, namely, pNZnisA-E3, pNZE3-spr1765, or pNZE3-spr1766, in GM17 were diluted 1:50 in minimal medium containing appropriate antibiotics and 0.5 ng/ml nisin (Sigma) for induction. Cultures were grown for 24 h at 30°C. Subsequently, supernatants were separated from cells by centrifugation. Next, supernatants were filtered through a 0.2-µm filter (Millipore). Prior to purification on a 5-ml HiTrap SP cation exchange column (GE Healthcare) using fast protein liquid chromatography (FPLC; on an Äkta purifier; Amersham Biosciences), supernatants were diluted 1:1 with a 100 mM lactic acid solution and filtered through 0.2-µm filters. After passage of supernatant through a column, unbound compounds were washed away with 100 mM lactic acid. Elution was performed with 1 M NaCl in 50 mM lactic acid solution. The fractions containing prepeptides were concentrated and desalted with 50 mM Tris-HCl of pH 5.5 on Microcon columns (Millipore). Intact prepeptides and peptides without a leader sequence were analyzed with MALDI-TOF mass spectrometry and used for screening of antimicrobial activity.

**N-terminal sequence removal.** The N-terminal sequence from the FPLC-purified prepeptides preP<sub>n</sub>eA1 and preP<sub>n</sub>eA2 was removed by trypsin. Prepeptides were incubated for 2 h at 37°C with 20 µg/ml trypsin in 100 mM Tris-HCl buffer (pH 8) containing 10 mM CaCl<sub>2</sub>. Alternatively, to remove the leader from preP<sub>n</sub>eA2, 180 µl of the prepeptide was incubated for 30 min at 37°C with 20 µl

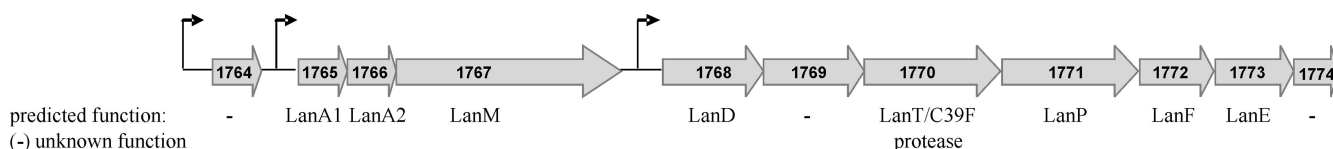


FIG. 1. Putative pneumococcin A1 and A2 gene cluster. Organization of the chromosomally located biosynthetic gene cluster of pneumococcin A1 and A2 genes (*spr1765* and *spr1766*, respectively) in *S. pneumoniae*. ORFs are represented by thick gray arrows, and their SPR gene identification numbers are shown in the gray arrows. The putative promoters are represented by thin and bent black arrows.

of 0.5 M phosphate buffer (pH 7.4) and with 10  $\mu$ l of leucine aminopeptidase (Sigma; suspension in 3.5 M ammonium sulfate).

**Mass spectrometry analysis.** To investigate whether chimeric peptides possess free cysteine residues, reactions with 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP) were performed. To obtain higher mass spectrum resolutions with MALDI-TOF, both prepeptides and propeptides, prior to CDAP treatment, were purified on a Hewlett Packard 1050 HPLC apparatus using a Jupiter Proteo C<sub>12</sub> (4- $\mu$ m, 90-Å, 250- by 4.6-mm) column. Reverse-phase purification was used with a gradient of 10% to 40% acetonitrile in purified water. All buffers contained 0.1% trifluoroacetic acid (TFA). The reactions with CDAP were performed as described previously (16, 35). Briefly, the pH of vacuum-dried trypsinated and nontrypsinated peptides and a control peptide, termed NisB2 (H-CRYTDPKPHIRLRK-OH), resuspended in 16  $\mu$ l MilliQ was adjusted to 2 or 3 with 0.1% TFA. Prior to treatment with CDAP, 1  $\mu$ l of a 100-mg/ml concentration of the reducing agent triscarboxyethyl phosphine (TCEP) was added to each mixture, and a reaction was carried out for 5 min at room temperature. Subsequently, 2  $\mu$ l of 100 mg/ml CDAP was added to the mixtures, and incubation followed for 15 min at room temperature.

Analysis was performed essentially as described before (19). Briefly, ZipTips (C<sub>18</sub> ZipTip; Millipore) were wetted with 100% acetonitrile and washed with 0.1% TFA. Subsequently, the supernatant containing the peptides was mixed with 0.1% TFA and applied to a ZipTip. Peptides that were bound to the column were washed with 0.2% TFA and eluted with 50% acetonitrile and 0.1% TFA. The eluent was mixed in a ratio of 1:1 with 10 mg/ml  $\alpha$ -cyano-4-hydroxycinnamic acid (matrix). A total of 1.5  $\mu$ l of this prepared mixture was spotted on the target and allowed to dry. Mass spectra were recorded with a Voyager-DE Pro (Applied Biosystems) MALDI-TOF. In order to increase the sensitivity, external calibration was applied with six different peptides (protein MALDI-MS calibration kit; Sigma).

**Amino acid sequence alignment.** Amino acid sequence alignment of nisin with pneumococcins A1 and A2 (SPR1765 and SPR1766, respectively) was performed with Clustal W (25), a program for multiple sequence alignments. The peptide sequences were derived from the NCBI database.

**Gel electrophoresis.** Prepeptides and mature peptides were analyzed on Tris-Tricine gels (44) and stained with Coomassie (Fermentas).

**Peptide concentration determination.** Peptide concentrations were determined using the DC protein assay of Bio-Rad. HPLC-purified nisin was used as a standard.

**MIC determination.** MIC assays for *M. flavus*, *S. pneumoniae*, *E. faecalis*, *S. aureus*, and *S. mitis* were performed in 96-well microtiter plates in GM17. The assay was performed as follows. Overnight cultures of the above-mentioned strains were diluted 1:50, and growth was continued to an optical density at 600 nm (OD<sub>600</sub>) of 0.2. Subsequently, 150- $\mu$ l cultures were mixed with 50  $\mu$ l of appropriate medium and various concentrations of peptides. The microtiter plates were incubated in a GENios (TECAN Benelux) at a suitable temperature for overnight growth of the strain, and the OD<sub>600</sub> was measured every 30 min. The MIC values were determined at the time when the cells without antimicrobial substance reached half of the maximal optical density. MICs were calculated from the lowest concentration of the antimicrobial substance that was able to inhibit the growth of the tested strain. All the susceptibility assays were performed in triplicate at least.

## RESULTS

**In silico analysis of the putative two-peptide lantibiotic-like cluster from *S. pneumoniae*.** After *in silico* analysis of 11 putative bacteriocin genes, identified by BAGEL (5), of *S. pneumoniae* R6 and their adjacent open reading frames (ORFs), we selected two of them, i.e., *spr1765* and *spr1766*. *In silico* analysis

of these two genes, as well as their nine adjacent ORFs, which most likely constitute a single cluster (Fig. 1), indicated that SPR1765 and SPR1766 belong to the class II two-component lantibiotics. We here propose to name SPR1765 and SPR1766 pneumococcins A1 and A2 (PneA1 and PneA2), respectively. So far there are no experimental data indicating that these bacteriocin-like peptides are expressed under laboratory growth conditions or in or under any other growth media or conditions we have tried (data not shown). *In silico* analysis showed that the nine adjacent genes (Fig. 1) are likely involved in pneumococcin A1 and A2 modification, transport, processing, and immunity. Gene *spr1767* encodes a protein with amino acid sequence similarity to a classic bifunctional LanM-like modification enzyme. The *spr1768* gene encodes a putative flavin adenine dinucleotide (FAD)-dependent flavoprotein that could catalyze the oxidative decarboxylation of C-terminal residues. The functions of SPR1764, SPR1769, and SPR1774 are unknown. The SPR1770 protein is a predicted ABC transporter containing putative N-terminal double-glycine peptidase activity (peptidase of C39 family) and is most likely responsible for transport of modified bacteriocins and for prepeptide processing. The SPR1771 protein shares 48% identity with NisP, the nisin leader peptidase. The last two genes of the regulon, *spr1772* and *spr1773*, encode a putative immunity protein and a putative ABC transporter, respectively. This analysis clearly shows that PneA1 and PneA2 very likely belong to the class II two-component lantibiotics.

Additionally, the gene cluster encoding the pneumococcins contains the gene encoding a putative flavoprotein (the *spr1768* gene product). The gene encoding this type of enzyme is also found in the gene clusters of epidermin and mersacidin. The modifications made by the flavoproteins are required for full activity of these peptides (24, 29). Whereas NisT displays rather broad transport specificity of peptides fused to the leader peptide of nisin, NisP has been shown to process only fully modified prenisin (19). Figure 2 shows the amino acid sequence alignment of pneumococcins A1 and A2 with the nisin structural peptide. There is a low similarity between the peptides both in the part of the leader sequence and in the part of the propeptide. On the basis of lantibiotic leader sequences (Fig. 2), the predicted pneumococcin A1 and A2 peptides possess three candidate cleavage sites, one behind GlyGly/GlyAla (after which SPR1770 might cleave), one behind a shared GlyAla sequence, and one behind ProArg. Strikingly, prenisin, prePneA1, and prePneA2 share the sequence GAXPRxT (the x being a variable residue), which comprises in the middle the site behind ProArg. These data, together with the shared 48% identity of the putative leader peptidase, SPR1771, with NisP, indicate that the Pne leader peptides end with ProArg.



PneA1	MTNFNSNEKFCGKSLKSLSADEMSLIYGASDGAEP	PRWTPTPIILKS---	AAASSKVCISA	57
PneA2	-----MKNDFVIGKSLKELSLLEEMQLVYGGTDGAD	PR---	STIICSA---TLSFIASYLGS	50
Nis	-----MSTKDFNLDLVSVSK-KDSGAS	PR-	ITSISLCTPGCKTGALMGCNMKT	46
		* . . . : . . .	. . . . .	
PneA1	AVSGIGGLVSYNNDCLG	74		
PneA2	AQTRCG--KDNKKK---	62		
Nis	ATCHCS--IHVSK----	57		
	* . . .			

FIG. 2. Amino acid sequence alignment of nisin with pneumococci A1 and A2. The cleavage site in the peptide sequence is highlighted in gray. Identical amino acid residues for all three sequences are indicated by an asterisk, conserved residues by a colon, and semiconserved amino acids by a point.

**Production, secretion, and purification of the chimeric peptides.** To investigate the production and secretion of chimeric peptides, pNZE3-spr1765 and pNZE3-spr1766 were introduced into *L. lactis* NZ9000 containing pIL3BTC. Cultures of *L. lactis* NZ9000 plasmid-containing derivatives induced with nisin were grown for 24 h in minimal medium. Subsequently, the supernatants were collected, and the prepeptides were purified on a HiTrap SP cation exchange column. The same procedure was applied for prenisin, a positive control. All prepeptides were produced and secreted, as visualized by Tris-Tricine electrophoresis (Fig. 3). Prepeptide concentrations were determined with HPLC-purified nisin of known concentration, as a reference. Without nisin induction, no secreted peptides were observed (data not shown). The production levels of both prePneA1 and prePneA2 were approximately 50% lower than that of prenisin.

**Streptococcal chimeric peptides are modified by nisin synthetase enzymes.** To test whether the purified chimeras prePneA1 and prePneA2 were modified, they were analyzed by MALDI-TOF spectrometry. Table 2 presents a summary of the obtained masses. Interestingly, analysis of prePneA1 and prePneA2 showed that both prepeptides were modified multifold. PrePneA1 showed four- and threefold dehydrations and prePneA2 four-, three-, and twofold. Chimeric prepeptides were processed by trypsin or leucine aminopeptidase and further characterized. Since the leader peptide keeps the prepeptide inactive, its removal allows the assessment of the antimicrobial activity of the mature peptides. Trypsin cleaves a peptide bond behind lysine or arginine, with arginine being preferred over lysine, allowing arginine-specific cleavage under controlled conditions (Table 2).

The prePneA1 was processed by trypsin, and only the nisin leader sequence was clipped off, leaving the mature peptide with multiple dehydrations (Table 2). However, prolonged digestion at a higher concentration of trypsin resulted in two additional mass peaks corresponding to two fragments (Table 2). The N-terminal part of PneA1 (Table 2) was cleaved off and

showed no dehydrations, likely due to protection against dehydration of Ser/Thr by their directly flanking residues (39). The identity of this N-terminal fragment was confirmed by sequence data obtained by postsourc decay (data not shown). The other lysine residue in the mature peptide (SSK) appears protected against proteolysis, likely due to posttranslational modifications in its vicinity (26). The five dehydrations that were observed with the mature PneA1 are therefore located in the C-terminal part of the peptide, in which six serines are present. Since one of those serines is located next to the lysine that is cleaved by trypsin, it is most likely that the observed dehydrations are all within the last 25 amino acids of PneA1, except for the first serine at SSK.

PneA2 contains a number of residues that are substrates for trypsin, and some of them are not protected by modified residues (Fig. 2). Therefore, we initially obtained a smaller fragment (Table 2, PneA2 fragment 2), which lacked the C-terminal extension but contained multiple dehydrated residues. Additionally, in HPLC-purified chimeric prePneA2, there was also a clear fraction containing a peptide fragment consisting of part of the nisin leader sequence and the N-terminal part of the PneA2 peptide (Table 2, prePneA2 fragment 1). Interestingly, this peptide fragment contained up to four dehydrations, which clearly shows that four out of five dehydrated residues are located in the first 11 amino acids of the mature peptide.

Subsequently, removal of the N-terminal leader peptide from chimeric peptides by NisP overexpressed in *L. lactis* was also investigated. However, neither the release of the leader peptide nor the antimicrobial activity of PneA1 or PneA2 was detected (data not shown). Intact PneA2 was obtained using leucine aminopeptidase and appeared to be three- to sixfold dehydrated (Table 2). Importantly, the activity of the leucine aminopeptidase apparently had stopped after the last Arg. This indicates the presence of a thioether bridge starting at either Ser1 or Thr2. Studies by Rink et al. indicated that flanking hydrophilic residues on both sides of a Ser or Thr would not favor dehydration. Furthermore, serines are less readily dehydrated than threonines (39). Therefore, a large extent of the dehydration of Ser1 flanked by Arg and Thr is not likely. Taken together, these findings indicate that Thr2 is fully dehydrated and is thioether cross-linked to Cys5.

To investigate whether thioether rings were formed, CDAP, which reacts only with free cysteines, was used. The control NisB2 peptide that contains one free cysteine was used as a positive control (not shown) for CDAP modification. CDAP modification converts a free thiol group of cysteine into an isothiocyanate, yielding a mass increase of 25 Da. The fivefold dehydrated PneA1 showed hardly any CDAP modification,

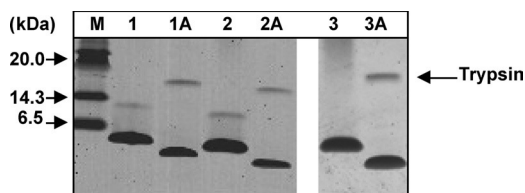


FIG. 3. Tris-Tricine gel illustrating trypsin-treated and non-trypsin-treated purified chimeric peptides and nisin. Lane M, marker; lane 1, prePneA1; lane 1A, trypsin-treated PneA1; lane 2, prePneA2; lane 2A, trypsin-treated PneA2; lane 3, prenisin; lane 3A, trypsin-treated prenisin.

TABLE 2. NisB-mediated dehydration of chimeric prepeptides and their fragments analyzed by MALDI-TOF mass spectrometry<sup>a</sup>

Peptide (fragment)	No. of observed dehydrations	Mass (M + H <sup>+</sup> ) without Met1 (Da)	
		Observed	Calculated
prePneA1 (nisin leader WTPTPIILKSAAASSKVCISAAVSGIGGLVSYNNDCLG)	4	6,009.6	6,009.9
	3	6,026.5	6,027.9
PneA1 (WTPTPIILKSAAASSKVCISAAVSGIGGLVSYNNDCLG)	6	3,655.9	3,658.3
	5	3,674.6	3,676.3
	4	3,692.8	3,694.3
	3	3,711.2	3,712.3
PneA1 (fragment 1 WTPTPIILK)	0	1,068.9	1,069.3
PneA1 (fragment 2 SAAASSKVCISAAVSGIGGLVSYNNDCLG)	4	2,643.6	2,644.0
	3	2,662.0	2,662.0
	2	2,680.5	2,680.0
prePneA2 (nisin leader STIICSATLSFIASYLGSAQTRCGKDNKKK)	4	5,437.6	5,437.2
	3	5,454.0	5,455.3
	2	5,472.2	5,473.3
PneA2 (STIICSATLSFIASYLGSAQTRCGKDNKKK)	6	3,086.7	3,085.6
	5	3,103.9	3,103.7
	4	3,121.5	3,121.7
	3	3,139.6	3,139.7
prePneA2 (fragment 1 SKKDSGASPRSTIICSATLSF)	4	2,085.1	2,084.7
	3	2,103.1	2,102.7
PneA2 (fragment 2 STIICSATLSFIASYLGSAQTR)	6	2,184.1	2,183.6
	5	2,200.8	2,201.6
	4	2,218.9	2,219.6
	3	2,236.7	2,237.6
	2	2,254.9	2,255.6
	1	2,273.0	2,273.6

<sup>a</sup> The average masses are shown.

indicating that there were no free cysteine residues and thus the formation of two thioether rings. The threefold dehydrated PneA1 peptide showed single and double CDAP modifications, indicating the formation of either one or no thioether

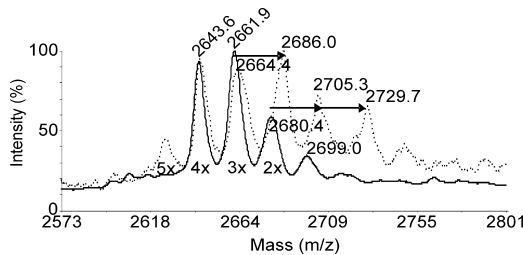


FIG. 4. Modified PneA1 contains thioether bridges. The SAAASS KVCISAAVSGIGGLVSYNNDCLG fragment dehydrated fourfold, threefold, and twofold (solid line) is treated with CDAP (dotted line) to detect the presence of modifiable cysteines (yielding peptide plus 25 Da) and nonmodifiable thioether linkage-forming cysteines. The 2,643.6-Da peak which is dehydrated fourfold shows a mild single CDAP addition. The 2,661.9-Da (threefold-dehydrated) peak shows single and double CDAP additions, as indicated by black arrows. The 2,680.5-Da (twofold-dehydrated) peak shows clear single and double CDAP additions; 1 CDAP, 2,705.3 Da (plus 25 Da), and 2 CDAP, 2,729.8 Da (plus 50 Da). The fivefold-dehydration peak is becoming visible after CDAP addition, indicating that this peptide has two thioether rings.

rings (Fig. 4). Similar studies of CDAP modification of PrePneA2 indicated the presence of two thioether rings in the extensively dehydrated peptides (not shown). These data demonstrate that the putative lantibiotics, which are entirely unrelated to nisin, can be successfully produced, modified, and secreted by the nisin synthetase machinery.

**The produced and modified peptides have significant antimicrobial activity.** To investigate the antimicrobial activity of the modified peptides, the chimeric prepeptides were incubated with trypsin or leucine aminopeptidase to remove the N-terminal leader sequence. Various dilutions of trypsin-treated peptides, namely, prenisin (positive control) and pneumococins A1 and A2, were tested for antimicrobial activity in the MIC assay (Table 3). Of all microorganisms tested, i.e., *M. flavus*, *S. pneumoniae*, *E. faecalis*, *S. aureus*, and *S. mitis*, only *M. flavus* was susceptible to the tested peptides, i.e., PneA1 and PneA2 (Table 3). In control experiments, no significant inhibition was found with either buffer or bovine serum albumin (BSA) treated with trypsin or with empty samples, i.e., fractions from prepeptide purifications that did not contain peptides (Table 3). Additionally, undigested chimeric prepeptides did not show significant antimicrobial activity against the indicator strain (Table 3). Unmodified PneA1 (MIC of >50  $\mu$ M) and PneA2 (MIC of >1.5 mM) propeptides obtained by chemical synthesis were at least 30-fold and 170-fold less active,

TABLE 3. Susceptibility of *M. flavus* to NisBC-modified PneA1 and PneA2 peptides

Sample	MIC ( $\mu$ M) for indicated digestion type <sup>a</sup> :		
	None	Trypsin	Leucine aminopeptidase
Prenisin	20	0.004	
Nisin leader peptide PneA1	80	0.6	
Nisin leader peptide PneA2	85	10	8.5
Nisin leader peptide PneA1 + nisin leader peptide PneA2	80	0.6	
BSA	NI	NI	
Buffer	NI	NI	
Empty sample	NI	NI	

<sup>a</sup> NI, no inhibition. MICs are calculated using the molecular weights of the mature peptides.

respectively, than the HPLC-purified active fraction of the corresponding NisBC-modified peptides, without leader peptide. This proves that NisBC-induced modifications are required for lantibiotic activity. PneA1 inhibited the growth of *M. flavus* at a peptide concentration of 0.6  $\mu$ M. PneA2, from which the leader sequence was removed by either trypsin or leucine aminopeptidase, inhibited growth at approximately 10 or 8.5  $\mu$ M, respectively (Table 3). The combination of both modified chimeric peptides, PneA1 and PneA2, did not act synergistically (Table 3). Thus, the data demonstrate that it is possible to utilize the nisin synthetase machinery for the production of antimicrobially active peptides unrelated to nisin.

## DISCUSSION

To the best of our knowledge, we present here for the first time the successful expression, modification, secretion, and biological activity of novel class II lantibiotics by the nisin synthetases, which normally produce nisin, a class I lantibiotic. To present a significant challenge as substrate peptides, pneumococcins A1 and A2 from *S. pneumoniae* R6, which presumably belong to the class II two-component lantibiotics, were chosen as substrates for the nisin enzymes. The class II two-component lantibiotics require a LanM-type enzyme that performs both dehydration and cyclization, whereas class I lantibiotics require LanB dehydratases and LanC cyclases. It has already been shown that LanBC-type enzymes can modify peptides other than nisin which are fused to the nisin leader sequence. Kluskens et al. and Kuipers et al. demonstrated that both medically relevant nonlantibiotic peptides and a truncated lantibiotic, lactacin 3147, that were fused with the nisin leader sequence, modified by NisB and NisC, were exported via NisT and contained dehydrated amino acids and lanthionine rings (16, 20). The same was proven by Rink et al. for various hexapeptides (38, 39). Thus, based on the discovery that the nisin synthetase machinery can accept various peptides as templates for modification, the propeptide part, which is the predicted maturing part of either the PneA1 or the PneA2 peptide, was fused to the nisin leader sequence and introduced into *L. lactis* that overexpresses NisBTC. The produced peptides were dehydrated multifold and contained thioether rings. Some dehydrated residues and one thioether ring could be

localized by studying peptide fragments and by applying leucine aminopeptidase.

To be biologically active, lantibiotics require prepeptide processing, i.e., removal of the leader sequence. The prepeptide sequences and the homology of the peptidase with NisP indicate that the site behind PR might be the processing site from which the propeptide starts. To liberate the mature peptides, we used trypsin or leucine aminopeptidase. Exported, purified, and processed peptides were tested for antimicrobial activity, and *M. flavus* was found to be highly susceptible to both the PneA1 and PneA2 peptides. Despite the fact that PneA1 and PneA2 are predicted two-component lantibiotics, we did not observe any significant synergistic effect when these peptides were combined (data not shown). The PneA1 and PneA2 peptides are a mixture of extensively modified and hardly modified peptides. These mixtures probably contain active, less active, and inactive peptides. Thus, the determined antimicrobial activity is the mean of the activities of all these peptides, indicating that the specific activity for a single active peptide might be higher. With respect to this, a preliminary experiment was performed with processed and NisBC-modified PneA1 and PneA2 peptides which were dehydrated four- and fivefold, separated, and purified from the total mixture. However, the MICs of both peptides were not significantly different from the MICs of the unpurified peptides (data not shown). A challenge for future work might be to sort out the active peptide fraction from the inactive fraction in order to get a better picture of which modifications yield antimicrobially active peptides.

The putative cluster of pneumococcins consists of 11 ORFs. In the cluster, two genes might be required for proper modification of PneA1 and PneA2. These genes encode a single putative LanM-type modification enzyme and a putative LanD-type flavoprotein. Flavoproteins catalyze the oxidative decarboxylation of a C-terminal cysteine residue involved in ring formation. A FAD-dependent flavoprotein catalyzes this reaction for mersacidin, a lantibiotic produced by *Bacillus* sp. (29). Another flavoprotein, which is flavin mononucleotide (FMN) dependent, catalyzes the same reaction for epidermin, a lantibiotic of *S. epidermidis*, and this enzyme is essential for formation of a biologically active peptide (24). It is not known whether the putative LanD-type flavoprotein of PneA1 and PneA2 performs a similar function in this cluster. Because the original cluster of pneumococcins contains LanM- and LanD-type modification enzymes, peptides modified by NisB and NisC might not be fully active by the lack of the oxidative decarboxylation. Furthermore, we do not know whether the native dehydration and ring pattern is exactly similar to the one installed heterologously by NisB and NisC. These factors might explain the presumably suboptimal antimicrobial activities and lack of synergism within this putative two-component lantibiotic system. However, both peptides, PneA1 and PneA2, still showed significant antimicrobial activity.

The production of nonlantibiotic or lantibiotic chimeras with a heterologous system has been reported using either closely related or nonlantibiotic peptides. For example, production of chimeric nonlantibiotic bacteriocin pediocin PA-1, which is fused to the leader of lactococcin A and/or to enterocin P, or enterocin A, which is fused to the leader of enterocin P, resulted in the secretion of active peptides (13, 30, 31). These



cases of successful production of biologically active bacteriocins concerns nonlantibiotic bacteriocins, which in contrast to lantibiotics do not require posttranslational modifications for antimicrobial activity. Production of class I lantibiotic chimeras, such as nisin/subtilin or subtilin/nisin, with either subtilin or nisin expression machineries was performed successfully (1, 23). Of the amino acid residues of the leaders and mature peptides of subtilin and nisin, 57% were identical (23). Studies using lactacin 481 synthetase demonstrated its ability to prepare other lantibiotics in the class II lactacin 481 family, including nukacin ISK-1, mutacin II, and ruminococcin A (37).

In contrast, we show here for the first time that it is possible to use the nisin synthetase system to produce, modify, and secrete lantibiotics, from a very different source and class, that exhibit considerable antimicrobial activity.

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